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(54) Title: METHOD FOR THE TRANSFER OF EXOGENOUS GENES IN PLANTS USING POLLEN AS A VECTOR

(57) Abstract

A method for the transfer of exogenous genes in flowering plants. Donor is isolated and incubated with pollen in a pollen-germinating medium. The pollen is then used to pollenate a compatable plant, and seed is collected. Offspring express traits from the exogenous DNA.

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METHOD FOR THE TRANSFER OF EXOGENOUS GENES IN PLANTS USING POLLEN AS A VECTOR

The invention herein described relates to a method for the transfer of exogenous genes in Angiosperms from a selected donor plant to a host plant. The method involves incubation of pollen from the parent plant with foreign DNA from the donor. The host plant is then pollinated with treated pollen and normal fertilization and development of seed occur. A self-pollination system is preferred. Transformed offspring generated from seed express genetic traits characteristic of the foreign DNA donor.

By way of background, it is generally known that major advances in the field of genetic engineering have occurred in recent years. Recombinant DNA technology has produced significant accomplishments, perhaps the most notable of which is the cloning and expression of eukaryotic genes in prokaryotic microorganisms. However, the functional expression of a novel gene in a genetically-engineering higher plant has not yet been reported (Barton, K.A., & Brill, W.J. 1983. Prospects in plant genetic engineering. Sci. 219: 671-675).

There are currently various strategies for introducing foreign genes into a given plant. Use of Agrobacterium tumefaciens and the Ti plasmid holds promise, but this system is limited to dicotyledonous plants. A. tumefaciens does not infect monocotyledonous plants. This plant group includes

grasses and cereals, and indeed most of the world's important food crops [Sci. Amer., 248(6):59, 1983]. Tissue culture techniques are also being investigated. Many dicotyledonous plants can be quite easily regenerated into intact plants from undifferentiated tissue-culture cells. However, the successful regeneration of monocotyledonous plants is rare and significantly more difficult to achieve. Classical plant breeding programs can be designed to transfer desired traits to host plants and develop new cultivars of economic importance, but this approach is a laborious and time-consuming process. Attempts to introduce genetic engineering procedures into plant breeding are further complicated by the fact that the genes for most important plant characteristics have not yet been identified.

In spite of the current limitations of various genetic engineering procedures, potential benefits to be gained from the development of effective plant transformation technology are of significant economic and social value. It should be possible to genetically engineer plants so as to provide advances in food production by: increasing yields; resistance to plant pests and pathogens; tolerance of cold, drought, salinity, mineral deficiency or toxicity; resistance to herbicides or pesticides; improved plant nutritional quality; incorporation of nitrogen-fixation capability, or other modes of decreasing fertilizer requirements. Such objectives of plant genetic engineering are formidable but not unrealistic goals in view of developing technology. Even if foreign genes can be introduced into certain desirable plants resulting in healthy genetically-modified plants, there is still the question of

whether or not such genes are expressed in a useful fashion.

When dealing with sexual reproduction among plants, alteration of the genome of either the haploid female gamete or the haploid male gamete can lead to expressed modifications of adult diploid progeny after normal pollination, fertilization and zygote development. The male gametophyte is a complex structure. As a representative example, the male gametophyte (pollen grain) of maize consists of a tube nucleus and a generative cell. Soon after germination the pollen tube protrudes from the pore of the pollen grain and the generative cell divides to produce two sperm. The pollen tube then enters the stigma, grows down the style, and enters the female gametophyte where it disposes of its contents into the cytoplasm of the embryosac [Pfahler, P.L. 1978. Biology of the male gametophyte. In D.B. Walden (ed.). Maize breeding and genetics. John Wiley and Sons, New York, pp. 517-530; Earle, E. 1982. Gametogenesis, fertilization and embryo development. In H. Smith and D. Grierson (eds.). Molecular biology of plant development. Bot. Monogs. 18: 285-305. Univ. Calif. Press, Berkeley; and Linskens, H.F. 1983. Pollination processes - Understanding fertilization and limits to hybridization. In W.J. Muedt (ed.), Strategies of plant reproduction. Beltsville Symposium 6: 35-50. Allenheld Osmun and Co., Tutowa]. A section directly behind the tip of the developing pollen tube lacks a cell wall [Cass, D.D., and D. J. Pateya. 1979. Growth of barley pollen tubes in vivo. Ultrastructural aspects of early tube growth in the stigmatic hairs. Can. J. Bot. 57: 386-396].

It is possible to produce genetically-modified progeny by modification of embryos resulting

from the sexual reproductive process. Microinjecting alien genes into embryos is a standard technique in genetically engineering the genomes of animals (Palmiter, R.D., et al. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300: 611-615). Some success in transforming plant embryos by microinjection of foreign DNA has also been achieved (Soyfer, U.N. 1980. Hereditary availability of plants under the action of exogenous DNA. Theor. Appl. Genet. 58: 225-235; Zhou, G., et al. 1983. Introduction of exogenous DNA into cotton embryos. Methods In Enzymology 101: 433-481). The size of DNA fragments that can become incorporated into the zygote or embryo genome is not known.

Mutations and transformations achieved through sexual transfer of exogenous DNA are phenotypically similar to expressions of known mutant loci. If actual gene transfer does take place, it is assumed that incorporation into the genome of the zygote will be at specific sites on one or more chromosomes (Rubin, G.M., and A,C, Spradling. 1982. Genetic transformation with transposable vectors. Sci. 218: 348-353; Spradling, A.C., and G.M. Rubin. 1982. Transposition of cloned P elements into Drosophila germ line chromosomes. Sci. 218: 341-347).

Transformed plants either segregate in a Mendelian fashion for specific marker genes in the second generation indicating single incorporation; or all second generation offspring are characterized by these specific marker genes indicating incorporation into several sites. More than one chromosome may thus receive the same DNA fragment. Standard genetic tests could be conducted to determine whether transformed phenotypes are genetically equivalent to known mutant

phenotypes, and whether loci coding for these genetically mutated phenotypes are located on the expected chromosomes and expected positions on chromosomes arms.

There is evidence to suggest that cultivated maize (Zea mays) arose through natural crossing; perhaps first with gamagrass (Tripsacum dactyloides). It is known that Zea mays (2n=20) crosses with Tripsacum dactyloides (L.) L. (2n=72) when the selected parents are not reproductively isolated by gametophytic barriers. Hybrids with 36 Tripsacum (Tr) + 10 Zea (Zm) chromosomes are characterized mostly by 18 Tr bivalents and 10 Zm univalents during meiotic prophase [de Wet, J.M.J. and J.R. Harlan. 1974. Tripsacum - maize interaction: A novel cytogenetic system. Genetics 78. 493-502; de Wet, J.M.J. et al. 1982. Systematics of Tripsacum dactyloides (Gramineae). Amer. J. Bot. 69: 1251-1257]. Four maize chromosomes occasionally associate with Tripsacum chromosomes and the Zea genome can become contaminated with Tripsacum genetic material (de Wet et al., 1972, cytology of maize - Tripsacum introgression. Amer. J. Bot. 59:1026-1029). When these hybrids are backcrossed with maize until plants with 2n=20 Zea chromosomes in Zea cytoplasm are recovered, the offspring are highly tripsacoid. Tripsacoid maize genotypes so produced carry several traits new to the genome of maize, and are highly desirable in maize improvement (Bergquist, R.L. 1981. Transfer from tripsacum dactyloides to corn of a major gene locus conditioning resistance to Puccinia sorghi. Phytopathology 71: 518-520). Intergeneric gene transfer requires a minimum of fifteen backcrosses. A technique is needed to transfer genes in fewer

generations, and without seriously disrupting the balanced genome of commercial maize inbreds.

Experiments suggest that alien pollen grains contribute to the genetics of the zygote without actually participating in fertilization (Pandey, K.K. 1978. Gametic gene transfer in Nicotiana by means of irradiated pollen. Genetica 9: 53-70; and Zhou, G., et al. 1979. The molecular basis of remote hybridization. Acta. Gen. Sinica 6: 405-413). Recent studies reveal that this is in fact possible [de Wet, J.M.J. et al. 1983. Counterfeit hybrids between Tripsacum and Zea (Gramineae). Amer. J. Bot. (in press)]. Diploid species of Tripsacum (2n=36) were pollinated by maize (2n=20). Diploid Tripsacum taxa produce functional female gametes that are haploid (18 chromosomes) or diploid (36 chromosomes). The cytologically non-reduced female gamete may function sexually or develop parthenogenetically to produce a functional embryo. Offspring from such crosses were therefore expected to have 18 Tr + 10 Zm, 36 Tr + 10 Zm, or 36 Tr + 0 Zm chromosomes, the last cytotype being maternal. These cytotypes were indeed produced, but some offspring with 36 Tr + 0 Zm chromosomes resembled true hybrids with 36 Tr + 10 Zm in phenotype. It was therefore argued that exogenous DNA introduced into the embryosac by Zea sperm was incorporated into the zygote without actual fertilization having taken place. Exogenous DNA introduced into the egg may also become incorporated into the genome of the zygote. Palmiter et al. (1982, supra) microinjected a recombinant DNA molecule consisting of a mouse promoter gene fused with a gene coding for the production of rat growth hormone into the pronuclei of fertilized mice eggs. Mice that developed from transformed eggs grew significantly larger than their

litter mates that were not genetically altered. Plant embryos were similarly transformed by Sayfer (1980, supra) and by Zhou et al. (1983, supra). Pollen may serve as a transfer vector of exogenous DNA (Hess, D. 1980. Investigations on the intra- and interspecific transfer of anthocyanin genes using pollen as vectors. Zeitschr. Pflanzenphysiol. Bd. 98: 321-337).

In light of the foregoing discussion of recent advances in the field of genetic engineering, and more specifically those developments related to agricultural plants, development of a vector system for the transfer of exogenous DNA transfer among economically important higher plants is highly desirable. Accordingly, an object of this invention is to provide a new and useful method for the transfer of foreign genes among flowering plants using the developing male gametophyte as a transfer vector. A further object is to provide a male gametophyte system for the transfer of genes between maize cultivars. Yet another object is to provide a method for the inter-species transfer of genes between gamagrass and maize using pollen as a vector. These and further objects are manifest in the following description and particularly delineated in the appended claims.

It has been discovered that the male gametophyte of Angiosperms can effectively act as a transfer vector of exogenous genes. One of species selected as experimental material for gene transfer is maize (Zea mays). Another experimental species is gamagrass (Tripsacum dactyloides). The genetics of maize is fairly well understood; stocks of marker genes are available; and two genes have been cloned and are available for experimentation. Using the technique herein described, pollen can be used as a transfer vector of foreign genes. The technique of

the invention can be used with flowering plants (Angiosperms) for dicot-dicot or monocot-monocot genetic transfer. This genetic engineering techniques is so simple that it can be used in plant breeding with little refinement. The dominant allele of the Rp1 locus conditioning resistance to the pathogen Puccinia sorghi Schw., as well as alleles of plant color genes, are successfully transferred from one maize inbred to another. Similarly, several traits are transferred from Tripsacum to Zea using the male gametophyte as a carrier of exogenous DNA. Transformation is not due to male gametophyte selection or conventional mutations. Either functional exogenous alleles replace existing alleles, or exogenous nucleotides become incorporated into existing loci, changing recessive alleles into their dominant counterparts.

The male gametophyte has two major advantages over the use of plasmids as transfer vectors. The most important advantage is efficiency. Germinating and incubating of pollen are readily achieved in the field, and self-pollination followed by selection are standard breeding tools for plant improvement. The usefulness of this technique is further enhanced by the ability to transform zygotes, bypassing problems associated with generating functional plants from protoplasts. Data indicate that germinating pollen grains incubated with alien DNA affect fertilization, and induce directional mutations in the genome of the zygote which are expressed in the resulting offspring and their descendents. The technique of the invention can be used to consistently transfer selected marker or other desirable genes from a DNA donor plant to a recipient mother cultivar. However, the mechanisms involved in DNA uptake by the

pollen tube, transportation of alien DNA to the embryosac by the male gametophyte, and exogenous nucleotide incorporation into the genome of the zygote, as well as the genetics of transferred or mutated genes in the offspring of the recipient mother, are not yet well known.

The method of the invention comprises the isolation of exogenous DNA from a selected donor plant, removal of mature pollen from the chosen donor plant, germination of this pollen in pollen-germinating liquid medium, incubation of germinating pollen with the foreign DNA, pollination of the mother plant with treated pollen, fertilization of the eggs within mature embryosacs of the mother plant, maturation of the ovary, obtainment of seeds from mother plant and germination of same, and selection of transformed plants from the population obtained from said seeds. In a situation where self-pollination is inconvenient or inoperative, pollen from a compatible cultivar related to the mother plant can be treated with exogenous genes and used to pollinate the respective mother plant. The method is thus broadly applicable to self-pollination as well as cross-pollination plant systems.

Foreign genes (i.e., nuclear DNA) are isolated from actively-growing seedling leaves using published procedures (Marmur, J. 1961. A procedure for the isolation of deoxribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208-219; Kislev, N. and I. Rubenstein. 1980. Utility of Ethidium Bromide in the extraction from whole plants of high molecular weight maize DNA. Plant Physiol. 66:1140-1143; Murray, M.G. and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res.

8:4321-4325). When using the male gametophyte as a vector for foreign genes, the most difficult aspects of the procedure of the invention are the germination of pollen and the pollination step following incubation with the donor DNA. An aqueous pollen-germinating medium (PGM) comprising carbohydrate, calcium, and boron is used. PGM comprising approximately 15% sucrose, 0.03% calcium nitrate, and 0.01% borate is preferred. Mature pollen is sprinkled onto a thin layer of PGM. Most of the pollen will begin to germinate within approximately 15 minutes. The previously-prepared donor DNA is added to the germinating pollen grains after approximately 10% of the pollen grains have begun germination. At a PGM:buffer ratio of approximately 9:1, PGM is poured over germinating pollen and SSC buffer with DNA is added to give a final DNA concentration of approximately 4-5 g/ml. Pollination is then initiated immediately. The PGM/DNA mixture is then transferred to the stigmatic surface of a receptive female inflorescence. Pollinated flowers are protected from foreign pollen by shoot bags until the PGM evaporates and then are covered with brown paper bags. Fertilization eventually occurs, but embryo and endosperm development is reduced. This effect is due to a reduction of functional pollen and sperm. It is known that several pollen grains are essential for the development of a seed (Klyucharena, M.V. 1962. The participation of many pollen tubes in fertilization. Tr. Inst. Genet. Akad. Nauk. CSSR. 29:238-264). In maize, for example, as many a 10 pollen tubes enter a style and penetrate the embryosac, although only one sperm actually fertilizes the egg and another fuses with the polar cells. The sperm that fuses with the

polar cells may come from the same or different male gametophyte as the sperm that fertilizes the egg.

Various mechanisms are possible to explain the uptake and transportation by the male gametophyte (pollen grain). Pollen grains typically contain a tube nucleus and a generative cell. The haploid generative cell divides to form two sperm, the sperm travel down the pollen tube of a germinating pollen grain, traverses the stigmatic surface and the style of a mature female inflorescence, and eventually enters the ovary where one sperm combines in the fertilization process with the haploid egg cell. Gametic delivery results in deposition near the egg of two sperm, the vegetative nucleus and cytoplasm by each of several male gametophytes. This is accompanied by loss of sperm and egg cell wall components. Gametic fusion results in the transmission of nearly the total sperm cytoplasm and organelle complement to the egg. The one sperm plays a role in the development of endosperm.

A section directly behind the tip of the developing pollen tube lacks a cell wall (Cass, D.D., and D.J. Pateya. 1979. Growth of barley pollen tubes in vivo. I. Ultrastructural aspects of early tube growth in the stigmatic hairs. Can. J. Bot. 57:386-88-396). It is suggested that exogenous DNA enters the male gametophyte through this pore in the pollen tube. It is not known how DNA is transported to the female gametophyte. The cytoplasm of the male gametophyte may act as a carrier of exogenous DNA or DNA absorbed into the pollen tube may become incorporated into the sperm during the division of the generative cell. Behavior of the male gametophyte during and after incubation is poorly understood. Pollen tube growth continues during incubation with donor plant DNA,

normal stigma penetration and fertilization occurs, but embryo and endosperm development is greatly reduced. It is also suggested that a critical number of male gametophytes need to deposit their contents into the cytoplasm of the female gametophyte for successful seed development. Increase in quantity of treated pollen used in pollination increases the number of seeds produced.

Results obtained using the method of the invention and maize demonstrate that exogenous genes are incorporated into the genome of the zygote. When and exactly how this occurs is unknown. If the DNA is carried to the female gametophyte by the sperm, incorporation may either be directly from the sperm genome or indirectly from the sperm cytoplasm. It is also possible that DNA is transported as free fragments in the cytoplasm of the male gametophyte or sperm. Incorporation may then take place during division of the zygote to produce an embryo.

The Zea mays cultivar B73 was selected for various experiments using the method of the invention. The female inflorescence of the standard maize inbred B73 consists of some 500 individual ovules arranged in 8 rows of paired spikelets around a central rachis. Each ovule has its own style with a feathery stigma, and contains a single female gametophyte. Sytles grow to over 15 cm long. Pollen grains are large, and it is possible to pick up individual grains with a fine, moist human hair for transportation to the stigma. Pollen germination and pollen tube growth down the stigma can be followed using fluorescence microscopy. Pollen germination is not severely affected by PGM or DNA incubation but pollen tube growth is retarded and few sperm reach the female gametophyte. It is not yet known how many pollen grains on a stigma are required

to effect successful pollination, how many pollen tubes must enter the embryosac for successful production, or the fate of the cytoplasmic contents and sperm of superfluous male gametophytes.

Maize B73 is self-pollinated with pollen incubated with DNA obtained from Tripsacum dactyloides or DNA from other maize genotypes carrying specific marker genes using the method of the invention. Experiments indicate that tripsacoid traits, as well as specific genes of the maize DNA donor transferable to maize through sexual transfer of exogenous DNA are similar to those incorporated into the maize genome through introgression (de Wet, J.M.J. et al. 1978. Morphology of teointoid and tripsacoid maize (Zea mays L.). Amer. J. Bot. 65:741-747). Differences in restriction sites (Bgl1 and BamH1 digests) distinguish B73 and tripsacoid B73. These differences are obvious in EtBr-stained gels of restriction digests, as well as on autoradiographs of similar digests hybridized to a ^{32}P labeled rDNA clone from soybean. It is anticipated that similar differences will characterize Tripsacum-DNA transformed maize.

The following non-limiting Examples further serve to illustrate the invention.

EXAMPLE 1

Procedure for Extraction of DNA from Donor Plants

Nuclear DNA is extracted from seedling or mature leaves of the donor genotype using a combination of published techniques [J. Mol. Biol. 3:208-219 (1961); Plant Physiol. 66:1140-1143 (1980); Nucl. Acids Res. 8: 4321-4325 (1980)], using a Trisbase buffer [0.2 M Trisbase (24.22g), 0.2 M Disodium di-H_2O EDTA

(74.45g), 4% SDS (40g) in one liter H_2O]. Extracted DNA is purified as described in procedures cited above. Pure DNA is stored in a few ml of SSC buffer [NaCl(8-77g) Na Citrate (4.412G) in one liter H_2O] at pH7.2 over chloroform at 4C. The DNA thus obtained is characterized by fragments showing a range of molecular weights (10^5-10^7 daltons) with a concentration at the high end of the scale.

EXAMPLE 2

Procedure for the Germination of Donor-Plant Pollen, Incubation with Exogenous Donor DNA, and Post-Incubation Pollination of the Mother Plant

Pollen germination and pollination after incubation with exogenous DNA are the most difficult aspects of the method of the invention using the male gametophyte as a carrier of foreign DNA. Pollen germinating medium (PGM) is prepared. PGM comprises a solution of approximately 15% sucrose, 0.03% calcium nitrate and 0.01% boric acid in water. Maize, as well as the pollen of other plants, germinates well in the PGM. The base of a large petri dish is covered with a thin layer of pollen germinating medium and sprinkled with mature pollen of the recipient mother. In experiments with maize approximately $27mm^3$ of pollen is used for each set of pollinations. In plants with lower pollen yield, and where individual flowers are pollinated, pollen from a single anther is sufficient to insure seed set. Depending on temperature (75 to 95°F in the field), pollen starts to germinate within 3 to 10 minutes. Approximately 60 to 90% of the pollen is germinated after 15 minutes.

DNA is obtained from donor plants according to the method of Example 1. Incubation of pollen with exogenous DNA begins after approximately 10% of pollen grains show visible signs of germination. Pollen tubes longer than the diameter of the grains break during pollination. Nine ml of PGM is poured over the germinated pollen and 1 ml of buffer with DNA is added, to obtain a DNA concentration of 4-5 g/ml. Pollination is initiated immediately.

The 11 ml of solution thus prepared is sufficient, for example, to pollinate three female inflorescences of corn each with approximately 300 to 500 ovules. Pollinating an ear of corn requires approximately one minute. Stigmas are cut to the tip of the cob twelve hours before pollination. The PGM with DNA and pollen is transferred to stigmas with a pasteur pipet. Pollinated ears are protected from foreign pollen by shoot bags until PGM evaporates, and they are then covered by standard brown paper bags. PGM takes approximately 15 minutes to evaporate. Incubation continues until the developing pollen tube enters the stigma, or until the DNA is destroyed on the stigmas. Pollen tube growth continues during incubation with DNA, and penetration of stigmas proceeds normally. Fertilization takes place, but embryo and endosperm development is greatly reduced. This is believed due to a reduction of functional pollen and sperm. Resultant seeds are then screened for transposed genetic characteristics. Similar procedures are adapted for other experimental plants.

EXAMPLE 3

The Effect on Seedset of Zea Mays B73 when Self-pollinated with DNA-treated Pollen

Maize inbreds B73, DP194 and Zm 1974 produce an average of 425, 368 and 572 caryopses respectively per female inflorescence when they are self-pollinated. Various treatments of mature pollen of B73 are performed using the methods described in Example 1 and 2. Results of these experiments are presented in Table I below:

TABLE I

Number of Female Inflorescences Within Seedset Classes of Self-Pollinated Maize Inbred B73 Using DNA-treated Pollen

Treatment[a]	Average no. caryopses/ear	Seedset classes								
		0	1-5	6-10	11-15	16-20	21-25	26-30	31-35	35
Selfed, no treatment	425									25
PGM + SSC buffer	5.1	14	7		3			1	2	2
PGM + Zm1974 DNA	1.2	55	26	2	1				1	
PGM + Ze2336 DNA	1.3	20	6		2					
PGM + Zd6905 DNA	0.8	88	22	1		1			1	
PGM + Td2762 DNA	1.4	37	11					2		

[a]Zm = Zea mays (domesticated maize); Z. mays subsp. parviglumis (teosinte; Zd = Z. diploperennis; Td = Tripsacum dactyloides.

All pollen treatments reduced seedset. Pollen grains germinated in PGM with SSC buffer before pollination resulted in an average seedset of five caryopses per inflorescence. About half of the ears pollinated produced no caryopses. When DNA was added to the PGM, average seedset was reduced to one caryopsis per inflorescence, with a majority of ears producing no caryopses. Increasing the number of pollen grains treated significantly increased percentage seedset. A majority of successful pollinations resulted in the formation of 1 to 5 well developed caryopses per inflorescence. Small caryopses with well developed embryos and poorly formed endosperm were sometimes produced. The maximum number of caryopses produced following DNA treatment was 50 per inflorescence.

EXAMPLE 4

The Effect on Seed Germination of Zea mays B73 When Self-Pollinated with DNA-treated Pollen

Maize inbreds B73 and DP194 germinate within 11 days under normal Illinois field conditions. Various treatments of mature B73 pollen are performed using the methods described in Examples 1 and 2. Results of these experiments are presented in Table II below.

TABLE II

Germination Eleven Days After Planting in 1983 Nursery, of Caryopses From Selfed Maize Inbred B73 With Various Pollen Treatments

Treatment	No. caryopses	No. germinated	% germination
Selfed, no treatment	87	79	90.8
PGM + SSC Buffer	18	11	61.1
PGM + Zm1974 DNA	218	47	21.6
PGM + Ze2336 DNA	121	43	35.5
PGM + Zd6905 DNA	209	47	22.5
PGM + Td2762 DNA	69	21	30.4

Zm = Zea mays (domesticated maize); Ze = Z mays subsp. parviglumis (teosinte); Zd = Z. diploperennis; Td = Tripsacum dactyloides.

In spring 1983, 90.8% of caryopses of untreated B73 germinated to produce healthy seedlings. Germination of B73 caryopses that resulted from DNA treated male gametophytes ranged from 21.6 to 35.5%. Buffer without DNA reduced germination to 61.1%. Treatment with DNA from different sources did not significantly change percentage germination. Experiments in 1981 and 1982 using inbreds Zm1974 and DP194 and the same DNA donors gave similar germination percentages. Replacing buffer with water did not improve germination.

Percentage seedset is negatively correlated with successful germination. Ears were classified

into those with 1 to 10, 11 to 20, 21 to 30 and 31 to 40 caryopses. Seedset classes were planted separately and percentage germination recorded. Results of this experiment are presented in Table III below.

TABLE III

Percent Germination of B73 Caryopses In Each Seedset Class, 30 Days After Planting In 1983 Nursery.

	No. caryopses per inflorescence			
DNA Donor	1-10	11-20	21-30	31-40
Zm 1974	41.2		34.1	3.5
Zm 2336	62.9	42.5		
Zm 6905	61.9	21.6		3.5
Td2762	39.4		30.8	

Zm = Zea mays (domesticated maize); Td = Tripsacum dactyloides

Seedset of 31-40 caryopses per ear resulted in 3.5% germination, of 21-30 caryopses in 31-34% germination, of 11-20 caryopses in 32-43% germination and 1-10 caryopses in 39-63% germination. Poor germination from ears with relatively high seedset is due to reduced amounts of endosperm in the small caryopses in relation to caryopses from ears with low seedset. Germination is essentially perfect when caryopses are planted in sterile vermiculite and kept in a growth chamber at 75 F. One hundred and three caryopses out of 105 planted, of inbred DP194 pollinated with DNA-treated pollen germinated in the

growth chamber. These include a random sample of caryopses from ears with 1-40 kernels. The same treatment gave 65% germination in field plantings. Seedlings were transplanted when the second leaf appeared. Transplanting started six days after sowing and continued for 29 days. At day eleven all seedlings of the control DP194, but only about 30% of the seedlings from treated plants were transplanted. A majority of seedlings that germinated after the sixteenth day were weak when they emerged, but eventually produced vigorous plants. Five seedlings were chlorotic and eventually died. Early seedling weakness prevented seedling emergence in field plantings.

EXAMPLE 5

Transfer of Rust Resistance in Zea mays Using the Male Gametophyte as a Gene Vector

Inbred Zea mays DP194 is highly susceptible to common leaf-rust caused by Puccinia sorghi. The DNA donor, Zea mays B14-A, is resistant to rust. Resistance is dominant over susceptibility, and the genotype of B14-A used as DNA donor was homozygous resistant (Rp1 /Rp1). Using the method described in Examples 1 and 2, various experiments are performed with cultivars of Zea mays. Results are presented in Table IV.

TABLE IV

Germination in Sterile Vermiculite at 75 F of 105 DP194 Caryopses Resulting From Treatment With DNA of B14-A Resistant to Rust

Days after planting	Seedlings transplanted	Observations
6	1-16	no. 3 robust
8	17-25	
10	26-30	no. 30 robust
12	31-43	no. 33 weak, died
14	44-60	no. 55 chlorotic, died
16	61-65	
18	66-70	
20	71-82	no. 74 rust resistant
		no. 75, 76 robust
22	83-90	no. 89 chlorotic, died
24	91-93	no. 92 weak, died
26	94-100	no. 94 robust, red cob
28	101	no. 101 weak, died
29	102-103	no. 102, 103 rust resistant
30	104-105	no. 104, 105 failed to germinate

Seedlings are transplanted when the second leaf appears, inoculated with rust starting at the 4-leaf stage. Field germination of the same treatment was 73%. DP194 control planted in vermiculite produced 90% germination within six days, and 100% germination by the eleventh day.

These data show that three out of 103 seedlings (No. 74, 102, 103) showed complete resistance after repeated inoculations with rust spores at the four-leaf and later stages. All other seedlings showed disease symptoms within five days after inoculation. Five out of 103 seedlings (No. 3, 30, 75, 76 and 94) were distinctly more vigorous than others, and developed into mature plants that were taller and more robust than the inbred. One of these robust plants was characterized by a red cob (rachis and glumes of the female inflorescence) and all were characterized by hairs along the leaf-sheath margins, two dominant characteristics of the B14-A DNA donor. True hybrids between B14-A and DP194 show comparable heterosis. That these robust plants and rust resistant plants could have resulted from accidental outcrossing is unlikely. Inbred DP194 was pollinated in a large hybrid seed production field with no B14-A or other maize resistant to P. sorghi around for approximately 2000 meters. Heterosis in DNA treated offspring suggests that a sufficient number of alien genes were introduced into the inbred via exogenous DNA to overcome inbreeding depression. Five seedlings (No. 33, 55, 89, 92, 101) showed deleterious effects of the treatment. They were weak or chlorotic and eventually died.

Example 6

Transfer of Characteristics Between Maize and Gamagrass Using the Male Gametophyte as a Gene Vector

Using the methods described above in Examples 1 and 2, Zea mays 1974 was self-pollinated with pollen incubated with Tripsacum dactyloides (gamagrass) DNA. This resulted in 277 caryopses, with

an average of 1.8 caryopses per ear. When these were planted in nurseries, 236 mature plants were obtained. Among these, 31 plants were obviously tripsacoid. Inbred Zm1974 produced a single culm, or rarely a second culm from one of the lower nodes. Twelve transformed plants were characterized by 4 to 6 tillers, a typically Tripsacum trait. Maize as well as Tripsacum are monecious. Maize is characterized by a terminal male inflorescence and lateral female inflorescence. In Tripasacum both lateral and terminal inflorescences are bisexual [de Wet, J.M.J. et al. Amer. J. Bot. 69:1251-1257 (1982)]. In Tripsacum the lower section of each inflorescence branch is composed of solitary female spikelets, alternately arranged in cavities of an indurated rachis, with the paired male spikelets arranged on the same rachis above the female section. Sixteen plants were characterized by solitary female spikelets on tassel branches below the male spikelets. Female spikelets in the tassel do occur in maize as a rare mutation, but they are paired as is typical in the female inflorescence of maize. Five of these robust plants tillered to produce 3 to 6 fertile culms. Peduncles of female inflorescences in Zm1974 vary from 13 to 57 cm in length. Among transformed offspring were three plants with peduncle lengths of 87 cm, 102 cm and 110 cm. Two of these plants tillered while the other was characterized by a single culm.

WHAT IS CLAIMED IS:

1. A method for the transfer of exogenous genes between embryo-forming plants using pollen as a vector comprising the steps of:

(a) obtaining DNA from a selected donor plant and optionally placing said DNA in a buffer and/or storing same for a period of time;

(b) removing mature pollen from a chosen pollen-donor plant;

(c) germinating said pollen in a pollen-germinating medium (PGM);

(d) incubating said germinating pollen with said donor DNA;

(e) pollinating said pollen-donor plant or other compatible mother plant with said treated pollen;

(f) collecting the resultant seed from said pollinated plant;

(g) germinating said seed and selecting transformed progeny expressing a characteristic or characteristics of said donor plant; and

(h) propagating said transformed plants.

2. A method according to Claim 1 wherein said embryo-forming plants are Angiosperms.

3. A method according to Claim 2 wherein said embryo-forming plants are mocotyledonous plants.

4. A method according to Claim 1 wherein said embryo-forming plants are dicotyledonous plants.

5. A method according to Claim 3 wherein said embryo-forming plants are graminaceous field crops.

6. A method according to Claim 2 wherein said exogenous DNA is from seedling leaves of donor plants.

7. A method according to Claim 6 wherein said buffer for said exogenous DNA is SSC having a pH of approximately 7.2.

8. A method according to Claim 7 wherein said PGM comprises an aqueous solution of a pollen-growth-stimulating medium containing carbohydrate, calcium, and boron.

9. A method according to Claim 8 wherein said PGM comprises approximately 5-25% sucrose, .01-0.09% calcium nitrate, and 0.003-0.03% borate.

10. A method according to Claim 8 wherein said PGM comprises approximately 15% sucrose, 0.03% calcium nitrate, and 0.01% borate.

11. A method according to Claim 10 wherein said germinating pollen is treated with donor DNA to obtain a DNA concentration of approximately 4 to 5 g/ml.

12. A method according to Claim 11 wherein said embryo-forming plants are cultivars of Zea mays.

13. A method according to Claim 11 wherein said DNA donor plant is Tripsacum and said mother plant is maize.

14. A method according to Claim 11 wherein said DNA donor plant is a cultivar of Zea mays and said mother plant is also a cultivar of Zea mays.

15. A method according to claim 14 wherein said donor plant is domesticated Z. mays, Z. mays subsp. parviglumis, or Z. diploperennis; and said mother plant is Zea mays B73.

16. A method according to Claim 13 wherein said donor plant is Tripsacum dactyloides and said mother plant is Zea mays B73.

17. A method according to Claim 14 wherein said donor plant is Z. mays B14-A and said mother plant is Z. mays DP194.

18. A method according to Claim 17 wherein said expressed characteristic is resistance to rust.

19. A chimeric DNA product comprising exogenous DNA from a donor plant and DNA from a recipient plant obtained by the steps comprising:

(a) obtaining DNA from a selected donor plant and optionally placing said DNA in a buffer and/or storing same for a period of time;

(b) removing mature pollen from a chosen pollen-donor plant;

(c) germinating said pollen in a pollen-germinating medium (PGM);

(d) incubating said germinating pollen with said donor DNA;

(e) pollinating said pollen-donor plant or other compatible mother plant with said treated pollen; and thus

(f) obtaining the resultant chimeric DNA product.

20. A chimeric DNA product according to Claim 19 wherein said product is contained in an embryo-forming plant.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/01774

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] A01B 76/00
US CL. 47/58

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/172.3, 317, 253 47/58 935/33, 35, 52, 63

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [6]

Lexis Computer Search-Chem Abstracts, Biosis, Life Sciences Collection

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N Holl et al. "Genetic Transformation in Plants in Tissue Culture and Plant Science 1974 H.E. Street, Editor, Academic Press, NY pp. 301-327	1-18
Y	N Hess et al 1976 Chemical Abstracts Vol 84 Abstract No. 102447c. "Investigations on the tumor induction in Nicotiana glauca by pollen transfer of DNA isolated from Nicotiana langsdorff	1-18
Y	N Pandey 1983 Chemical Abstracts Vol 99 Abstract No. 173046s "Evidence for gene transfer by the use of sublethally irradiated pollen in Zea mays and theory of occurrence by chromosome repair through somatic recombination and gene conversion".	1-18
Y	N Hess 1980 Chemical Abstracts Vol 93 Abstract No. 164546d "Investigations on the intra-and interspecific transfer	1-18

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
05 January 1985	28 JAN 1985
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	of anthocyanin genes using pollen as vectors"	1-18
Y	N Harlan et al 1977 "Pathways of Genetic transfer from Tripsacum to Zea mays Proc. Natl Acad Sci(USA) Vol 74 No. 8 pp 3494-3497	1-18
Y	N Hess 1981 "Attempts to Transfer Kanamycin Resistance of Bacterial Plasmid Origin in Petunia hybrida using Pollen as Vectors" Biochem. Physiol. Pflanzen Vol 176 pp 322-328	1-18